PATENT
Docket No. 229752001500

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Anthony Soljanich

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In the application of:

Charles Andrew COLLYER et al.

Serial No.: 09/980,370

Filing Date: May 17, 2002

For: METHOD OF PROPHYLAXIS AND TREATMENT AND AGENTS USEFUL FOR SAME

Examiner: L. Mayes

Group Art Unit: 1653

RESPONSE TO RESTRICTION REQUIREMENT

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

This is in response to the Office Action dated February 7, 2003 (Paper No. 10) for which a response was due on March 7, 2003 and for which a two (2) month extension of time is also requested to extend the time for response from March 7, 2003 to May 7, 2003.

Applicants respectfully traverse the Examiner's grounds for restriction. The Examiner cites Bramanti *et al.* (J. Bacteriol. 175: 7413-7420 (1993) in support of her argument that Bramanti *et al.* teach agents that antagonize interactions between a molecule from a microorganism having an HA2 domain and an HA2-binding motif on a porphyrin-containing molecule and therefore there is no novel inventive step common to all claims.

Applicants submit that Bramanti *et al.* do not characterize OMP26 as a protein with an HA2 domain. Bramanti *et al.* do not provide any sequence data for OMP26 and therefore it was not possible to know which peptides or combinations of peptides in Bramanti *et al.* were involved in the reported interactions. Further, the polyclonal antibodies that purportedly link their study to OMP26 were not characterized in terms of specificity by Bramanti *et al.*

In a subsequent publication from the same group, Kim, Chu and Holt (*Microbial Pathogenesis* 21: 65-70 (1996); submitted herewith) disclose a partial sequence of another hemin-binding cell surface protein (now known as HmuY) and state that Bramanti *et al.* could not sequence OMP26 due to N-terminal blockage (*see* Kim *et al.* at page 68, second full paragraph).

Applicants submit that at the time of its publication Bramanti *et al.* had not established whether OMP26 was a HA2 protein. While it may seem obvious in hindsight, it is the Applicants' invention that specifically provides, for the first time, functional characterization of the HA2 polypeptide. Therefore, the subject matters of the claims in Groups I and II are linked by a common element of novelty under PCT Rule 13.1. Applicants respectfully request reconsideration of the restriction requirement.

In the event that Applicants' arguments are not found persuasive by the Examiner, and in response to the restriction requirement, Applicants would elect Group I (claims 1-11, 18 and 19), with traverse. Applicants expressly reserve their right under 35 U.S.C. § 121 to file a divisional application directed to the non-elected subject matter during the pendency of this application, or an application claiming priority from this application.

Applicants respectfully request examination of the claims on the merits.

In the unlikely event that the transmittal letter is separated from this document and the Patent Office determines that an extension and/or other relief is required, Applicant petitions for any required relief including extensions of time and authorizes the Assistant Commissioner to charge the cost of such petitions and/or other fees due in connection with the filing of this document to **Deposit Account No. 03-1952** referencing docket no. 229752001500. However, the Assistant Commissioner is not authorized to charge the cost of the issue fee to the Deposit Account.

Respectfully submitted,

Dated: May 7, 2003

By: 

Shantanu Basu
Registration No. 43,318

Morrison & Foerster LLP
755 Page Mill Road
Palo Alto, California 94304-1018
Telephone: (650) 813-5995
Facsimile: (650) 494-0792

Microbial Pathogenesis 1996; **21**: 65–70

Short communication

Isolation and characterization of a hemin-binding cell envelope protein from *Porphyromonas gingivalis*

Sung-Jo Kim, L. Chu, and S. C. Holt*

Department of Microbiology, University of Texas Health Center at San Antonio, San Antonio, TX 78284, U.S.A.

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Kim, S.-J. (Department of Microbiology, University of Texas Health Science Center at San Antonio, San Antonio, TX 78284, U.S.A.), L. Chu and S. C. Holt. Isolation and characterization of a hemin-binding cell envelope protein from *Porphyromonas gingivalis*. *Microbial Pathogenesis* 1996; **21**, 65–70.

A 30 kDa (heated 24 kDa) hemin-binding protein whose expression is both hemin and iron regulated was identified and purified in *Porphyromonas gingivalis* 381. A strong hemin-binding function was found by LDS-PAGE and TMBZ staining when cells were grown under hemin (iron)-limited conditions. N-terminal amino acid sequence analysis of CNBr-digested 24 kDa hemin binding protein revealed that this protein belongs to a new, so far undescribed hemin-binding class of proteins.

Key words: Hemin; *Porphyromonas gingivalis*; TMBZ; outer membranes.

Introduction

While there are several studies which have dealt with the expression of hemin-regulated proteins in the oral pathogen, *Porphyromonas gingivalis*,[1–5] there are few studies which have demonstrated actual hemin-binding. Smalley *et al.*[5] identified a major tetramethylbenzidine(TMBZ) staining 32 kDa (unheated) protein in hemin-limited *P. gingivalis* W50, while Grenier[6] demonstrated that the hemin-binding property of *P. gingivalis* ATCC 33277 is mediated by the lipopolysaccharides, particularly the Lipid A region. In the study presented here, we have identified, purified, and characterized a putative hemin binding protein in *P. gingivalis* 381.

Results

Identification of hemin-binding protein

In Fig. 1(a), the upregulation of the 56 and 30 kDa proteins (unheated) is very clear. TMBZ staining [Fig. 1(b)] revealed the only protein to bind the stain was at 30 kDa when cells were grown under hemin(iron) restriction. Note in Fig. 1(b) that

* To whom correspondence and reprint requests should be addressed.

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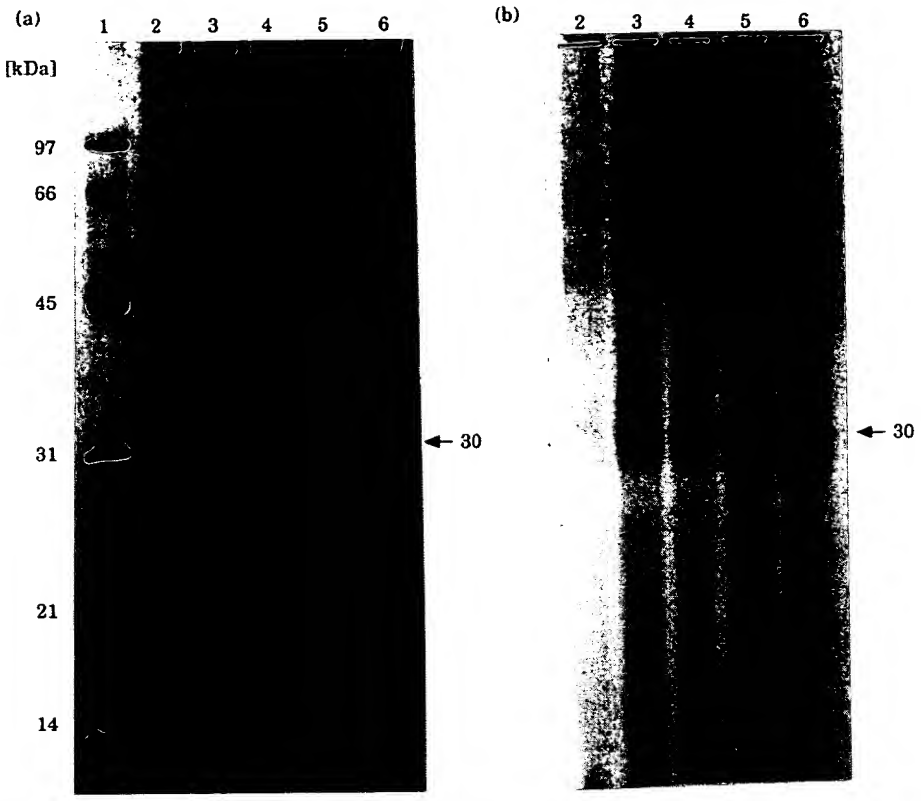

Fig. 1. Coomassie Brilliant blue stained (a) and tetramethylbenzidine (TMBZ) stained (b) LDS-PAGE of cell envelopes from *P. gingivalis* 381. Protein (70 μg) was applied to each lane following incubation with hemin. Lane 1, low mol.wt. standards; lane 2, cells grown + 7.7 μM hemin; lane 3 passage 5; lane 4, cells grown + 200 μM BPD; lane 5, cells grown + 300 μM BPD; lane 6, cells grown + 400 μM BPD.

with increasing iron restriction (i.e. 300, 400 μM BPD), there was increased TMBZ binding. A series of very lightly staining TMBZ bands might correspond to LPS.

Purification of hemin-binding protein

The 30 kDa *P. gingivalis* 381 hemin-binding protein was sequentially purified from the unheated cell envelopes (Fig. 2). In Fig. 2, lane 2, the cell envelope fraction contained numerous cell envelope proteins, including LPS-associated proteins. 1% CHAPS solubilization resulted in the removal of a large number of membrane proteins and associated LPS. The 30 kDa protein in Fig. 2, lane 3 was the major protein. The 30 kDa protein was isolated from the SDS-PAGE gels of the 1% CHAPS-soluble fraction (Fig. 2, lane 4). This protein was aggregated with proteins at 24 and 56 kDa. Heating of the isolated 30 kDa protein resulted in the appearance of the 24 kDa protein and several other minor and weakly staining proteins (Fig. 2, lane 5). The 24 kDa protein was isolated from the SDS-PAGE of this heated 30 kDa protein (Fig 2, lane 6). The resulting 24 kDa protein was isolated from the gel as a single protein band, with no contaminating proteins even when the gels were overloaded with large amounts of purified protein (data not shown).

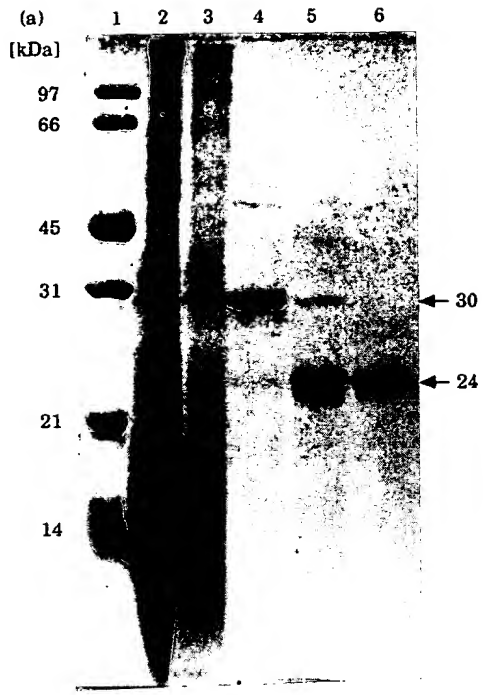

Fig. 2. SDS-PAGE analysis of the purification of the 30 kDa (unheated; 24 kDa heated) hemin-binding protein from *P. gingivalis* strain 381. Lane 1, low mol.wt. standards; lane 2, cell envelope fraction; lane 3, 1% CHAPS-soluble fraction; lane 4, isolation of 30 kDa protein; lane 5, 100°C, heated isolation 30 kDa protein; lane 6, purified 24 kDa protein.

Table 1 Cyanogen bromide fragmentation of 24 kDa hemin-binding protein from *Porphyromonas gingivalis* 381

Fragments	Amino acid sequence*
20 kDa	DQATSVPTDG(X)Y(X)TVD(X)KLGRITVK
17 kDa	GPDG(H)ZMEYEE
12 kDa	EYEEQGFSEVITGKKNAQGFAA(X)G(X)LEF(S)

*(X), unknown; (), assume to be correct amino acid.

N-terminal sequence analysis

The CNBr digestion of 24 kDa hemin-binding protein revealed at least three polypeptide bands (data not shown). Internal amino acid sequence analysis of one of these fragments (12 kDa) is seen in Table 1.

Discussion

Bacterial growth and membrane protein expression which are regulated by iron (hemin) have been reported in several microorganisms).[7-11] Many of these proteins have been implicated as functional components of iron (hemin) uptake systems

in these species. Although the requirement for hemin has been known for many years,[12] little is known about the mechanism(s) by which *P. gingivalis* binds and uptakes hemin into the cell. There have also been no reports of the purification and characterization of an actual hemin-binding protein from *P. gingivalis.*

Several Gram negative bacteria are known to utilize hemin as a sole source of iron. Hemin-binding proteins have been identified in several of these species, including *Shigella flexnerii,*[13] *Bacteroides fragilis,*[10] *Neisseria gonorrheae,*[14] *Hemophilus influenzae,*[15–17] *Treponema denticola,*[7] and *P. gingivalis.*[5,18] However, only few of these proteins have been purified and characterized. In the study presented here, we were able to establish that a 30 kDa (unheated) cell envelope associated protein from *P. gingivalis* strain 381 bound hemin and was stained with TMBZ. The expression of this protein appeared to be tightly regulated by the level of hemin(iron) in the growth medium.

Functionally, Omp 26 of Bramanti and Holt[18] and Omp 32 of Smalley *et al.*[5] appear similar with respect to hemin. Bramanti and Holt[18] were unable to sequence Omp 26 because of N-terminal blockage, and Smalley *et al.*[5] did not provide any sequence data for their Omp 32. Internal amino acid sequence analysis of the CNBr digested fragment and a search of GenBank for proteins with similar internal amino acid sequence to the 24 kDa protein revealed no significant similarities, and we consider the 30 kDa (heated 24 kDa) membrane protein to represent a newly described hemin binding protein from *P. gingivalis* strain 381. To our knowledge, the study described here is the first to identify, purify and biochemically characterize a hemin-binding protein from *P. gingivalis.* Work is in progress to further characterize the molecular structure of this protein.

Materials and methods

Bacterial strain and culture conditions. *P. gingivalis* 381 was grown anaerobically on the surface of enriched Trypticase soy agar, or in 2.1% (w/v) Mycoplasma broth base (BBL, Becton Dickinson, Cockeysville, MD) supplemented with 1 μg/ml menadione and 5 μg/ml hemin. Plate grown cultures were routinely incubated for 4 days and used as the inoculum for liquid growth. Liquid grown cells were incubated for approximately 24 h, equivalent to late exponential growth phase. For hemin restriction (i.e. hemin starvation), late exponential or early stationary phase cultures were grown with excess hemin (i.e. 7.7 μM hemin), and serially passaged at least 5 times as a 10% inoculum into hemin-free medium. Iron limitation was achieved by the addition of 100 to 400 μM of the iron-chelating compound, 2,2-bipyridyl (BPD., Sigma Chemical Co., St. Louis, MO), into liquid growth medium containing 7.7 μM hemin. All glassware was washed in chromic acid and rinsed in deionized water to remove contaminating iron and hemin. Culture purity was assessed by Gram staining and plating to solid medium.

Cell envelope preparation. Cells were harvested by centrifugation at 12,000 × ***g***, for 20 min at 4°C, washed three times in cold phosphate-buffered saline (PBS, pH 7.2), and resuspended in PBS containing a protease inhibitor cocktail consisting of 2 mM each of phenylmethylsulfonyl fluoride (PMSF), benzamidine and Na-P-tosyl-L-lysine chloromethyl ketone (TLCK). Cell envelopes were prepared by French pressure cell disruption of whole cells in PBS (pH 7.2) by four 15,000 lb/in^2 disruption cycles. The cell envelopes were removed after low-speed (10,000 × ***g***, 30 min) and high-speed (2,000,000 × ***g***, 2 h) centrifugation. Protein concentration was determined using the bicinchoninic acid (BCA) assay of (Pierce, Lockford, IL).

Polyacryamide gel electrophoresis. The discontinuous sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) procedure of Laemmli[19] was employed for determination of protein distribution, and lithium dodecyl sulfate (LDS) PAGE was used

for the TMBZ staining studies. 12% acrylamide separating gels were routinely used. All gels were run with a 4% acrylamide stacking gel in a vertical slab gel apparatus (Hoefer Scientific, San Francisco, CA). Proteins were visualized by Coomassie Brilliant Blue-R-250 stain (CBB). The hemin-associated peroxidase activity of the cell envelope protein was determined by tetramethylbenzidine (TMBZ, Sigma Chemical Co., St. Louis, MO) staining of LDS-PAGE gels. The TMBZ staining was carried out as described by Stugard *et al.*[13]

Purification of hemin-binding protein. Isolation of hemin-binding protein from *P. gingivalis* 381 cell envelope was accomplished by solubilization in the zwitterionic detergent 3 [(3-chloramidopropyl)-dimethyl-ammonio] 1 propane sulfonate (CHAPS; Pierce, Rockford, IL). Cell envelopes from *P. gingivalis* 381 (passage 5) were isolated as described above, and solubilized by the addition of CHAPS to a final concentration of 1% (v/v) and incubated at 37°C, 1 h. The resulting suspension was ultracentrifuged at 100,000 ***g*** for 1h to pellet insoluble material, and the CHAPS-soluble fraction was either used immediately for the purification of hemin-binding protein or stored at −20°C until used. The hemin-binding protein referred to as 'unheated 30 kDa' (see Results) from *P. gingivalis* 381 was purified from the CHAPS-soluble membrane fraction of hemin-starved passage five cells by 1D-SDS-PAGE through a 12% gel employing a preparative comb with one reference well. The elution protocol of Hager and Burgess[20] was used. Purity of the isolated protein was confirmed by 1D SDS-PAGE.

Cyanogen bromide digestion and N-terminal sequencing. Initial experiments revealed that the unheated 30 kDa protein did not transfer well to a polyvinylidine difluoride (PVDF) membrane (Pro Blot, Applied Biosystems, Foster City, CA). Heating the 30 kDa protein at temperatures above 70°C resulted in the modification of the protein to a molecular weight of 24 kDa. In this heated, denatured condition the protein transferred quantitatively, and was used for N-terminal amino acid analysis. Attempts to directly sequence the N-terminus of the 24 kDa protein were unsuccessful because of a blocked N-terminus. Therefore, sequences were determined after cyanogen bromide (CNBr) digestion.

Cleavage by CNBr was carried out on the acetone precipitated 24 kDa protein. Several crystals of CNBr were added to 100 μl of 70% formic acid and swirled to dissolve. 50 μl of this solution was added to the acetone precipitated sample and allowed to proceed in the dark at room temperature, 16 h. The digest was dried under an N_2 stream and reduced to dryness in a SpeedVac SC 100 (Savant). The dried digest was dissolved in 1 x treatment buffer for SDS-PAGE, heated at 100°C for 5 min, electrophoresed through an exponential gradient gel (7.5 to 20%), and electroblotted to a PVDF membrane at 100 mA, 4 h. After transfer, the ProBlot membrane was removed from the transblotting sandwich and rinsed with deionized water. Protein bands on the ProBlot membrane was visualized by Amido black staining. The protein bands were excised from the dried membrane, and its N-terminal amino acid sequence was determined with an Applied Biosystems (Foster City, CA) Model 477A gas–liquid phase sequenator coupled to an on line high-performance liquid chromatography model 120A analyser.

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